

## DNA DAMAGE-INDUCED APOPTOSIS: INHIBITION BY CALMODULIN ANTAGONIST, FAS RECEPTOR ANTIBODY AND CASPASE INHIBITORS

R. Ray, B. J. Benton, M. E. Burke, T. Rockwood, K. R. Bhat, D. R. Anderson, J. P. Petrali, and  
W. J. Smith

US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010

P. Ray

Walter Reed Army Institute of Research, Silver Spring, MD 20910

D. S. Rosenthal

Georgetown University School of Medicine, Washington, D.C. 20007

### ABSTRACT

Sulfur mustard (HD, bis-(2-chloroethyl) sulfide) is a vesicant that causes DNA strand breaks and apoptosis in cultured normal human epidermal keratinocytes (NHEK). HD causes apoptosis via two independent pathways, a  $\text{Ca}^{2+}$ /calmodulin (CaM)-mediated mitochondrial pathway and Fas receptor (CD95) pathway. We studied the effects of the exogenously added CaM antagonist W7, the CD95 antibody, the caspase-3 inhibitor Ac-DEVD-CHO, and the general caspase inhibitor Z-VAD-fmk on NHEK viability loss (Calcein AM fluorescence assay, LDH release assay) due to HD. All protected against HD. Z-VAD-fmk was the most effective. These results provide a logical approach toward developing an anti-apoptotic vesicant countermeasure.

### INTRODUCTION

In the skin, the basal epidermal keratinocytes are the most sensitive targets of the vesicant sulfur mustard (HD). Therefore, in these cells, understanding the mechanisms of HD cytotoxicity and its prevention should be useful in developing medical countermeasures against HD. Previous studies have demonstrated that HD causes apoptosis in cultured normal human epidermal keratinocytes (NHEK) indicated by caspase-3 activation and PARP (poly (ADP-ribose) polymerase) cleavage (Rosenthal *et al.*, 1998). HD-induced apoptosis in NHEK involves two independent pathways: (a)  $\text{Ca}^{2+}$ /calmodulin (CaM)-dependent mitochondrial pathway and (b) Fas receptor (CD95) pathway. Both the mitochondrial and the CD95 pathways act essentially via a common caspase cascade responsible for the apoptotic endpoints. In this project, we studied the protection against cell viability loss due to HD by (a) the CaM antagonist W7, (b) a CD95 antibody preparation, (c) the caspase-3 inhibitor Ac-DEVD-CHO, and (d) the general caspase inhibitor Z-VAD-fmk. The results are analyzed to propose a prospective pharmacological antivesicant intervention.

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE <b>01 JUL 2003</b>		2. REPORT TYPE <b>N/A</b>		3. DATES COVERED <b>-</b>	
4. TITLE AND SUBTITLE <b>Dna Damage-Induced Apoptosis: Inhibition By Calmodulin Antagonist, Fas Receptor Antibody And Caspase Inhibitors</b>				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010</b>				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>					
13. SUPPLEMENTARY NOTES <b>See also ADM001523.</b>					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT <b>UU</b>	18. NUMBER OF PAGES <b>6</b>	19a. NAME OF RESPONSIBLE PERSON
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>			

## EXPERIMENTAL METHODS

HD ( $\geq 98\%$  pure) was obtained from the Soldier Biological and Chemical Command (SBCCOM), Aberdeen Proving Ground, MD, and was used for cultured NHEK exposure as described previously (Ray *et al.*, 1995). Frozen stocks of NHEK (passage 2) and liquid culture medium were obtained from the Clonetics Corporation (San Diego, CA). Cells were cultured in appropriate tissue culture vessels (150 or 75 cm<sup>2</sup> flasks, 24-well plates) as described previously (Ray *et al.*, 1995). A crude preparation (4 mg protein/ml phosphate buffered saline) of the monoclonal antibody against human Fas antigen (CD95) was obtained by 40% ammonium sulfate fractionation from the culture medium of HB-11726 cells grown as recommended by the supplier, ATCC. Other chemicals and reagents were of analytical or tissue culture grade as applicable.

Cell viability was determined biochemically by lactate dehydrogenase (LDH) release (Ray *et al.*, 1995) and calcein AM fluorescence (Molecular Probes manual for Live/Dead Viability/Cytotoxicity Kit) assays. In the latter assay, live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Caspase-3 assay was conducted using the synthetic tetrapeptide fluorogenic substrate Ac-DEVD-AMC and the caspase-3 specific tetrapeptide inhibitor Ac-DEVD-CHO, both obtained from BD PharMingen (Rosenthal *et al.*, 1998). Protein was determined using the Biorad protein assay reagent and bovine  $\gamma$ -globulin as the standard.

## RESULTS AND DISCUSSION

Exposure of cultured NHEK to HD (300  $\mu$ M, 16 hr) results in both a decrease in calcein fluorescence and an increase in LDH release compared with HD-unexposed NHEK, indicating cell viability loss due to HD. Under the same conditions specified above, these indicators of cell viability loss are accompanied by caspase-3 activation, which supports that HD-induced cell death involves apoptosis. Either a CaM antagonist or the CD95 antibody prevents HD-induced cell viability loss. This observation suggests that intervention against HD may be possible via either the mitochondrial or the CD95 pathway. Inhibitors of caspases that are common mediators of both the mitochondrial and the CD95 pathways of apoptosis also prevent HD-induced cell viability loss. Of the two types of caspase inhibitors tested, the apparently more potent general caspase inhibitor Z-VAD-fmk may be a prospective antivesicant compound.

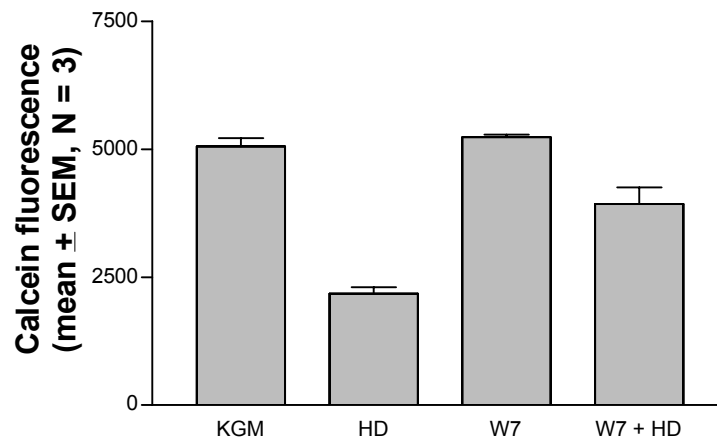


Figure 1. The CaM antagonist W7 (50  $\mu$ M), added to NHEK culture medium 30 min prior to HD (300  $\mu$ M), prevents HD-induced cell viability loss.

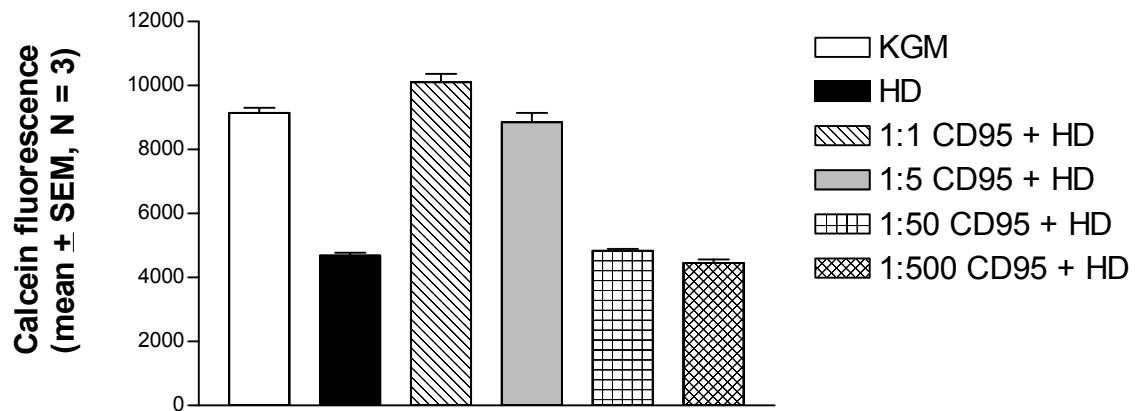


Figure 2. Addition of the CD95 (Fas receptor) antibody preparation to NHEK culture medium 30 min prior to HD (300  $\mu$ M) protects against HD-induced cell viability loss in a dilution-dependent manner.

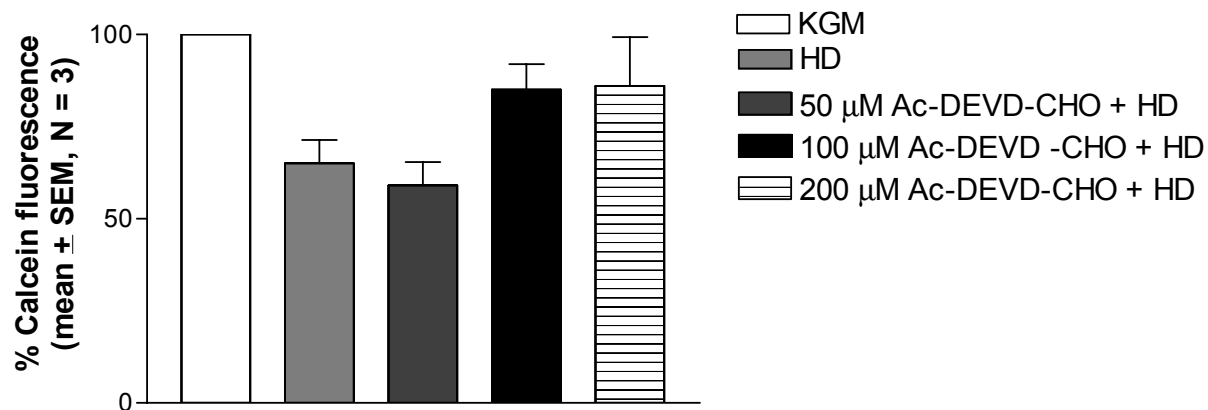


Figure 3. The caspase-3 inhibitor Ac-DEVD-CHO, added prior to HD (300  $\mu$ M), prevents HD-induced cell viability loss with a maximal effect at 100  $\mu$ M.

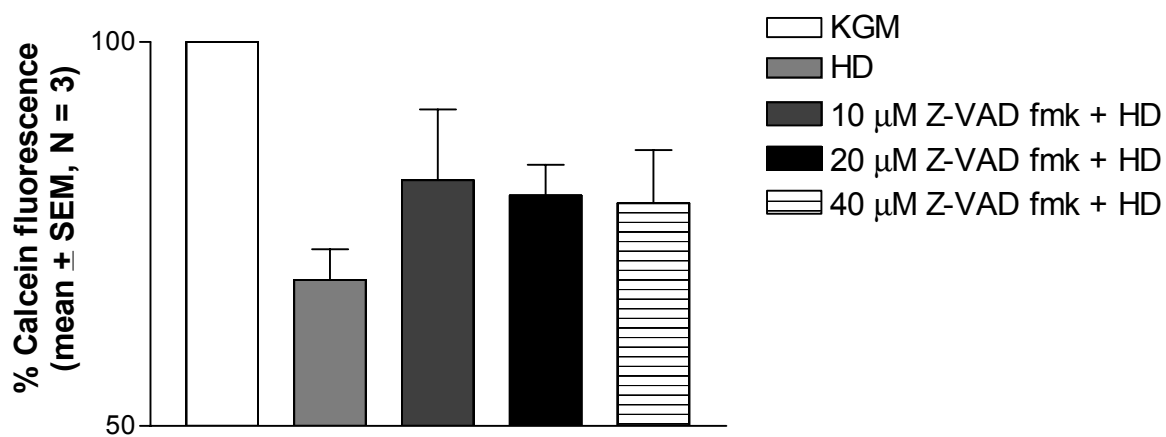


Figure 4. The general caspase inhibitor Z-VAD-fmk, added prior to HD (300  $\mu$ M), prevents HD-induced cell viability loss with a maximal effect at 10  $\mu$ M.

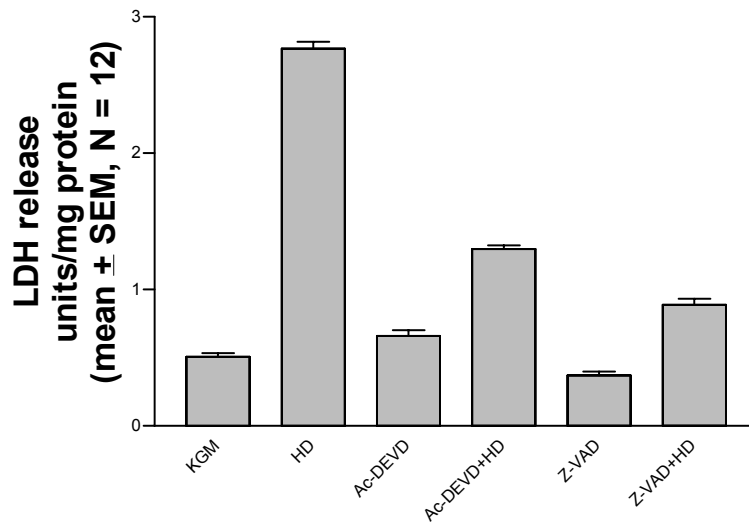


Figure 5. Either caspase-3 inhibitor Ac-DEVD-CHO (100  $\mu$ M) or the general caspase inhibitor Z-VAD-fmk (10  $\mu$ M) prevents HD (300  $\mu$ M)-induced lactate dehydrogenase (LDH) release from cultured NHEK, Z-VAD-fmk apparently being more potent than Ac-DEVD-CHO.

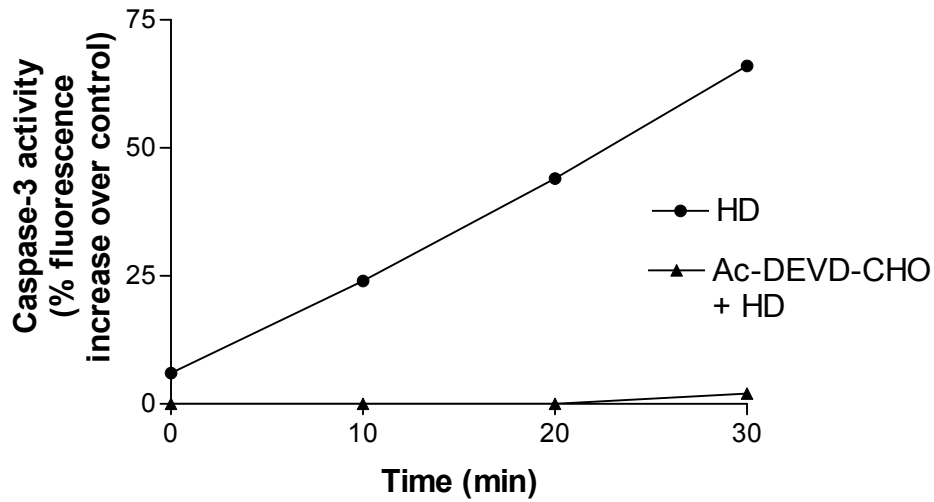


Figure 6. Cell viability loss (calcein fluorescence decrease, LDH release) due to HD (300  $\mu$ M, 16 hr) is accompanied by caspase-3 activation, an indicator of apoptosis.

## CONCLUSIONS

There are reports in the literature that in the cultured normal human epidermal keratinocytes (NHEK) model, sulfur mustard (HD) causes apoptosis or programmed cell death via mechanisms involving calmodulin (CaM), Fas (CD95) receptors, and caspases. The results presented in this report show that CaM antagonist W7, CD95 receptor antibody, and the general caspase inhibitor Z-VAD-fmk prevent HD-induced cell death. We propose that these agents may be useful as pharmacological antidotes to protect against the skin injury (vesication) due to HD.

## REFERENCES

1. Ray, R., Legere, R.H., Majerus, B.J., and Petralli, J.P.: Sulfur Mustard-Induced Increase in Intracellular Free Calcium Level and Arachidonic Acid Release From Cell Membrane, *Toxicol. Appl. Pharmacol.*, **131**: 44-52, 1995.
2. Rosenthal, D. S., Rosenthal, C. M. G., Iyer, S., Smith, W., Ray, R., and Smulson, M. E. Sulfur mustard induces terminal differentiation and apoptosis in keratinocytes via a  $\text{Ca}^{2+}$ -calmodulin and apopain-dependent pathway. *J. Invest. Dermatol.*, **111(1)**: 64-71, 1998.